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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

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Si aucun titre n'est indiqué se referer à la description.)

Improved vaccine

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The present invention relates to improved vaccines, especially viral vaccines and methods of making thereof.

Host protection from invading pathogens involves cellular and humoral effectors and results from the concerted action of both non-adaptive (innate) and adaptive (acquired) immunity. The latter is based on specific immunological recognition mediated by receptors, is a recent acquisition of the immune system, and is present only in vertebrates. The former evolved before the development of adaptive immunity, consisting of a variety of cells and molecules distributed throughout the organism with the task of keeping potential pathogens under control.

B and T lymphocytes are the mediators of acquired antigen-specific adaptive immunity, including the development of immunological memory, which is the main goal of creating a successful vaccine. Antigen presenting cells (APCs) are highly specialized cells that can process antigens and display their processed fragments on the cell surface together with molecules required for lymphocyte activation. This means that APCs are very important for the initiation of specific immune reactions. The main APCs for T lymphocyte activation are dendritic cells (DCs), macrophages, and B cells, whereas the main APCs for B cells are follicular dendritic cells. In general DCs are the most powerful APCs in terms of initiation of immune responses stimulating quiescent naive and memory B and T lymphocytes.

The natural task of APCs in the periphery (e.g. DCs or Langerhans cells) is to capture and process antigens, thereby being activated they start to express lymphocyte co-stimulatory molecules, migrate to lymphoid organs, secrete cytokines and present antigens to different populations of lymphocytes, initiating antigen-specific immune responses. They not only activate lymphocytes, under certain circumstances, they also tolerize T cells to antigens.

Antigen recognition by T lymphocytes is major histocompatibility complex (MHC)-restricted. A given T lymphocyte will recognize an antigen only when the peptide is bound to a particular MHC molecule. In general, T lymphocytes are stimulated only in the presence of self MHC molecules, and antigen is recognized only as peptides bound to self MHC molecules. MHC restriction defines T lymphocyte specifity in terms of the antigen recognized and in terms of the MHC molecule that binds its peptide fragment.

Intracellular and extracellular antigens present quite different challenges to the immune system, both in terms of recognition and of appropriate response. Presentation of antigens to T cells is mediated by two distinct classes of molecules ~ MHC class I (MHC-I) and MHC class II (MHC-II), which utilize distinct antigen processing pathways. Mainly one could distinguish between two major antigen processing pathways that have evolved. Peptides derived from intracellular antigens are presented to $CD8^+$ T cells by MHC class I molecules, which are expressed on virtually all cells, while extracellular antigen-derived peptides are presented to $CD4^+$ T cells by MHC-II molecules. However, there are certain exceptions to this dichotomy. Several studies have shown that peptides generated from endocytosed particulate or soluble proteins are presented on MHC-I molecules in macrophages as well as in dendritic cells. Therefore APCs like dendritic cells sitting in the periphery, exerting high potency to capture and process extracellular antigens and presenting them on MHC-I molecules to T lymphocytes are interesting targets in pulsing them extracellularily with antigens in vitro and in vivo.

The important and unique role of APCs, including stimulating activity on different types of leukocytes, is reflecting their central position as targets for appropriate strategies in developing successful vaccines. Theoretically one way to do so is to enhance or stimulate their natural task, the uptake of antigen(s). Once pulsed with the appropriate antigens the vaccine is directed

against, APCs should start to process the uptaken antigen(s), thereby being activated, expressing lymphocyte co-stimulatory molecules, migrating to lymphoid organs, secreting cytokines and presenting antigens to different populations of lymphocytes thereby initiating immune responses.

Activated T cells generally secrete a number of effector cytokines in a highly regulated fashion, e.g. interleukin 2 (IL-2), IL-4, IL-5, IL-10 and interferon-gamma (IFN-g). The functional detection of cytotoxic T lymphocyte responses to specific antigens (e.g. tumor antigens, in general antigens administered in a vaccine) is commonly monitored by an ELISpot assay (enzyme-linked immunospot assay), a technique analyzing cytokine production at the single cell level. In the present invention an ELISpot assay for the cellular immunity (type 1 immune response) promoting cytokine IFN-g is used to monitor successful antigen-specific T cell activation. Furthermore, the cytokine IL-4 is determined as an indicator for a type 2 response, usually involved in promoting strong humoral responses. In addition, the humoral immune response was determined by ELISA (IgG1 as indicator for a type 2 response, IgG2b as indicator for a type 1 response).

It has previously been shown that polycations efficiently enhance the uptake of MHC class I-matched peptides into tumor cells, a peptide or protein pulsing process which was called "TRANSloading". Furthermore, it has been shown that polycations are able to "TRANSload" peptides or proteins into antigen presenting cells in vivo as well as in vitro. In addition, co-injection of a mixture of poly-L-arginine or poly-L-lysine together with an appropriate peptide as a vaccine protects animals from tumor growth in mouse models. This chemically defined vaccine is able to induce a high number of antigen/peptide-specific T cells. That was shown to be at least partly attributable to an enhanced uptake of peptides into APCs mediated by the polycation indicating that APCs when pulsed in vivo with antigens can induce T cell-mediated immunity to the administered antigen.

As opposed to adaptive immunity, which is characterised by a highly specific but relatively slow response, innate immunity is based on effector mechanisms that are triggered by differences in the structure of microbial components relative to the host. These mechanisms can mount a fairly rapid initial response, which mainly leads to neutralization of the noxious agents. Reactions of innate immunity are the only defence strategy of lower phyla and have been retained in vertebrates as a first line host defence before the adaptive immune system is mobilised.

In higher vertebrates the effector cells of innate immunity are neutrophils, macrophages, and natural killer cells and probably also dendritic cells, whereas the humoral components in this pathway are the complement cascade and a variety of different binding proteins.

A rapid and effective component of innate immunity is the production of a large variety of microbicidal peptides with a length of usually between about 12 and about one hundred amino acid residues. Several hundred different antimicrobial peptides have been isolated from a variety of organisms, ranging from sponges, insects to animals and humans, which points to a wide-spread distribution of these molecules. Antimicrobial peptides are also produced by bacteria as antagonistic substances against competing organisms.

Two major subsets of $CD4^+$ T cells (T-helper 1 (Th1) and T-helper 2 (Th2)) have been identified in mouse and human, based on their secretion of different cytokine profiles and their different effector functions. Th1 cells are mainly involved in the generation of so called type 1 immune responses, which are typically characterised by the induction of delayed-type hypersensitivity responses, cell-mediated immunity, immunoglobulin class switching to IgG2a/IgG2b and secretion of i.a. Interferon-gamma. In contrast, Th2 cells are involved in the generation of so called type 2 responses, which are characterised by the

induction of humoral immunity by activating B cells, leading to antibody production including class switching to IgG_1 and IgE. Type 2 responses are also characterized by the secretion of the following cytokines: IL-4, IL-5, IL-6 and IL-10.

In most situations, the type of response induced (type 1 or type 2) has a significant impact on the protective efficacy of a vaccine. Alternative adjuvants tend to favour specific types of responses. However, adjuvant selection is complicated by functional unpredictabilities and also by commercial constraints and availability.

Infections with Influenza virus belong to the most important and frequent infections and has a significant mortality rate, especially for older people or people with deficiencies in the immune system. Currently, there are a number of Influenza vaccines on the market; however, not all vaccinations lead to protectivity against Influenza infections. Therefore, a need to improve current Influenza vaccines exists in order to enlarge the protection efficacy.

Moreover, since most of the current vaccines are almost exclusively eliciting type 2 responses, also a need exists to provide improved vaccines which show a type 1 directed immune response or vaccines which allow - in addition to a type 2 response - also a significant type immune reaction. Moreover, vaccines already available should be provided in an improved form which allows the induction of a type 1 response.

Therefore, the present invention provides an improved vaccine against (viral) infections comprising an antigen, a peptide of the formula R_1-XZXZ$_N$XZX-R_2 and an immunostimulatory deoxynucleic acids containing deoxyinosine and/or deoxyuridine residues.

According to the experiments performed in course of the present invention, the combination of these two types of Immunizers has shown a synergistical effect with respect

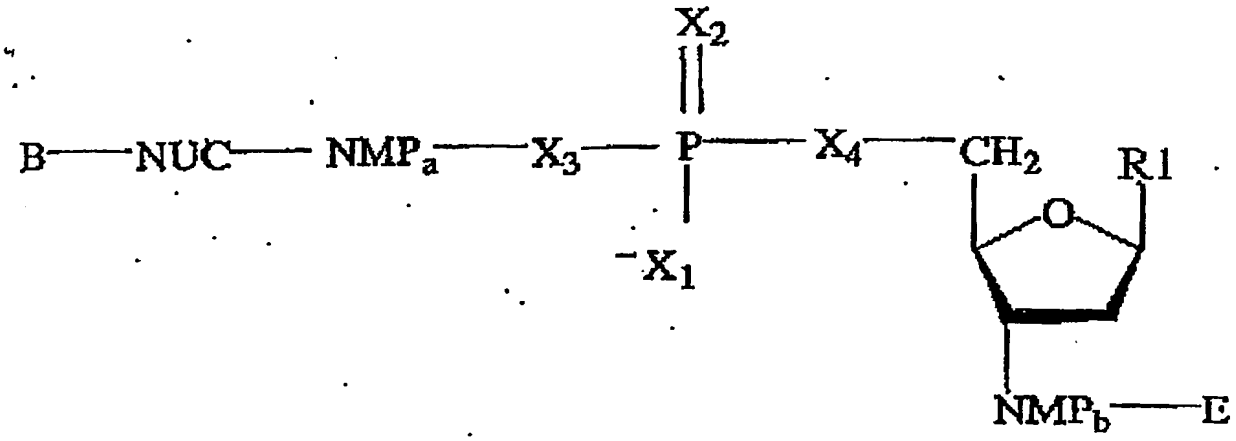
to antigens. This was specifically shown with respect to common Influenza antigens (especially haemagglutinin and neuraminidase) and Hepatitis virus antigens. This synergistic effect especially for viral antigens was not derivable from the known properties of these substance classes. Although each of these two substance classes is known to have excellent immunostimulating properties (WO 02/32451, WO 01/93905 and PCT/EP02/05448), the combined effect for viral pathogens, especially for Influenza and Hepatitis virus antigens, was significantly better than could be expected from the mere addition of these single efficacies.

With the present invention it is also possible to significantly improve viral vaccines, especially Influenza or Hepatitis A, B or C vaccines, being already available or on the market simply by additionally providing the combination of the two types of Immunizers according to the present invention.

The present invention therefore provides a vaccine for preventing infections of influenza virus comprising

- an antigen, especially a viral antigen,
- a peptide comprising a sequence R_1-XZXZ$_N$XZX-R_2, whereby N is a whole number between 3 and 7, preferably 5, X is a positively charged natural and/or non-natural amino acid residue, Z is an amino acid residue selected from the group consisting of L, V, I, F and/or W, and R_1 and R_2 are selected independantly one from the other from the group consisting of -H, -NH_2, -$COCH_3$, -COH, a peptide with up to 20 amino acid residues or a peptide reactive group or a peptide linker with or without a peptide; X-R_2 may be an amide, ester or thioester of the C-terminal amino acid residue of the peptide (in the following also referred to as "Peptide A") and

- an immunostimulatory oligodeoxynucleic acid molecule (ODN) having the structure according to the formula (I)



wherein
R1 is selected from hypoxanthine and uracile,
any X is O or S,
any NMP is a 2' deoxynucleoside monophosphate or monothiophosphate, selected from the group consisting of deoxyadenosine-, deoxyguanosine-, deoxyinosine-, deoxycytosine-, deoxyuridine-, deoxythymidine-, 2-methyl-deoxyinosine-, 5-methyl-deoxycytosine-, deoxypseudouridine-, deoxyribosepurine-, 2-amino-deoxyribosepurine-, 6-S-deoxyguanine-, 2-dimethyl-deoxyguanosine- or N-isopentenyl-deoxyadenosine-monophosphate or -monothiophosphate,
NUC is a 2' deoxynucleoside, selected from the group consisting of deoxyadenosine-, deoxyguanosine-, deoxyinosine-, deoxycytosine-, deoxyinosine-, deoxythymidine-, 2-methyl-deoxyuridine-, 5-methyl-deoxycytosine-, deoxypseudouridine-, deoxyribosepurine-, 2-amino-deoxyribosepurine-, 6-S-deoxyguanine-, 2-dimethyl-deoxyguanosine- or N-isopentenyl-deoxyadenosine,
a and b are integers from 0 to 100 with the proviso that a + b is between 4 and 150, and

B and E are common groups for 5' or 3' ends of nucleic acid molecules (in the following also referred to as "I-/U-ODN").

Of course, the present vaccine may further contain other substances, e.g. suitable pharmaceutically acceptable diluents or carrier, buffer or stabilising substances, etc..

The vaccine according to the present invention may further contain additional adjuvants, especially an $Al(OH)_3$ adjuvant (Alum).

Alum, as meant herein includes all forms of Al^{3+} based adjuvants used in human and animal medicine and research. Especially, it includes all forms of aluminum hydroxide as defined in Römpp, 10th Ed. pages 139/140, gel forms thereof, aluminum phosphate, etc..

This is especially preferred for vaccines which are already on the market and contain such $Al(OH)_3$ adjuvants. In such a case, the combination of Immunisers according to the present invention may simply be added to such an existing vaccine.

The present antigen is preferably a viral antigen. If pronounced (or exclusive) Th1 type 1 responses should be specifically necessary, T cell epitopes (see introduction above) are preferred as antigens. Preferably the antigen is a viral antigen. In the example section the present invention is proven in principle and specifically effective with influenza and hepatitis viral antigens, namely with the hepatitis B surface antigen, which are preferred antigens according to the present invention.

Of course, the pharmaceutical preparation may also comprise two or more antigens depending on the desired immune response. The antigen(s) may also be modified so as to further enhance the immune response.

Proteins or peptides derived from viral or bacterial pathogens, from fungi or parasites, as well as tumor antigens (cancer vaccines) or antigens with a putative role in autoimmune disease may be used as antigens (including derivatized antigens like glycosylated, lipidated, glycolipidated or hydroxylated antigens). Furthermore, carbohydrates, lipids or glycolipids may be used as antigens themselves. The derivatization process may include the purification of a specific protein or peptide from the pathogen, the inactivation of the pathogen as well as the proteolytic or chemical derivatization or stabilization of such a protein or peptide. Alternatively, also the pathogen itself may be used as an antigen. The antigens are preferably peptides or proteins, carbohydrates, lipids, glycolipids or mixtures thereof.

According to a preferred embodiment T cell epitopes are used as antigens. Alternatively, a combination of T cell epitopes and B cell epitopes may also be preferred.

Also mixtures of different antigens are of course possible to be used according to the present invention. Preferably, proteins or peptides isolated from a viral or a bacterial pathogen or from fungi or parasites (or their recombinant counterparts) are used as such antigens (including derivatized antigens or glycosylated or lipidated antigens or polysaccharides or lipids). Another preferred source of antigens are tumor antigens. Preferred pathogens are selected from human immunodeficiency virus (HIV), hepatitis A and B viruses, hepatitis C virus (HCV) or other Flaviviridae, such as Japanese encephalitis virus (JCV), rous sarcoma virus (RSV), Epstein Barr virus (EBV) Influenza virus, human papilloma virus (HPV), Rotavirus, Staphylococcus aureus, Chlamydia pneumonias, Chlamydia trachomatis, Mycobacterium tuberculosis, Streptococcus pneumonias, Bacillus anthracis, Vibrio cholerae, Plasmodium sp. (Pl. falciparum, Pl. vivax, etc.), Aspergillus sp. or Candida albicans.

In the case of peptide antigens the use of peptide mimotopes/agonists/superagonists/antagonists or peptides changed in certain positions without affecting the immunologic properties or non-peptide mimotopes/agonists/superagonists/antagonists is included in the current invention. Peptide antigens may also contain elongations either at the carboxy or at the amino terminus of the peptide antigen facilitating interaction with the polycationic compound(s) or the immunostimulatory compound(s).

Antigens may also be derivatized to include molecules enhancing antigen presentation and targeting of antigens to antigen presenting cells.

The Influenza or Hepatitis antigen to be used according to the present invention is not generally restricted to a specific form, it seems that the effect according to the present invention is even further pathogen-specifically enhanced for Influenza or Hepatitis B, but not specific for a certain type of antigen from this Influenza or HBV pathogen. However, it is preferred to use the standard Influenza or HBV antigens also in the present vaccines, i.e. a haemagglutinin antigen, a neuraminidase antigen, a combined antigen or a combination of one or more of these antigens.

Preferably, proteins or peptides isolated from an Influenza virus or HBV source (e.g. a cell culture) or their recombinant counterparts are used as such antigens, including derivatized antigens.

The vaccine according to the present invention preferably further (or, specifically in the case of Influenza, HCV or HBV, even instead of the Peptide A) contains a polycationic peptide.

The polycationic peptides or compound to be used according to the present invention may be any polycationic compound which shows the characteristic effect according to the WO 97/30721. Preferred

polycationic compounds are selected from basic polypeptides, organic polycations, basic polyaminoacids or mixtures thereof. These polyaminoacids should have a chain length of at least 4 amino acid residues. Especially preferred are substances containing peptidic bounds, like polylysine, polyarginine and polypeptides containing more than 20%, especially more than 50% of basic amino acids in a range of more than 8, especially more than 20, amino acid residues or mixtures thereof. Other preferred polycations and their pharmaceutical compositons are described in WO 97/30721 (e.g. polyethyleneimine) and WO 99/38528. Preferably these polypeptides contain between 20 and 500 amino acid residues, especially between 30 and 200 residues.

These polycationic compounds may be produced chemically or recombinantly or may be derived from natural sources.

Cationic (poly)peptides may also be polycationic anti-bacterial microbial peptides. These (poly)peptides may be of prokaryotic or eukaryotic origin or may be produced chemically or recombinantly. Peptides may also belong to the class naturally occurring antimicrobial peptides. Such host defense peptides or defensives are also a preferred form of the polycationic polymer according to the present invention. Generally, a compound allowing as an end product activation (or down-regulation) of the adaptive immune system, preferably mediated by APCs (including dendritic cells) is used as polycationic polymer.

Furthermore, also neuroactive compounds, such as (human) growth hormone (as described e.g. in WO01/24822) may be used as immunostimulants (Immunizers).

Polycationic compounds derived from natural sources include HIV-REV or HIV-TAT (derived cationic peptides, antennapedia peptides, chitosan or other derivatives of chitin) or other peptides derived from these peptides or proteins by biochemical or recombinant production. Other preferred polycationic compounds are cathelin or related

or derived substances from cathelicidin, especially mouse, bovine or especially human cathelicidins and/or cathelicidins. Related or derived cathelicidin substances contain the whole or parts of the cathelicidin sequence with at least 15-20 amino acid residues. Derivations may include the substitution or modification of the natural amino acids by amino acids which are not among the 20 standard amino acids. Moreover, further cationic residues may be introduced into such cathelicidin molecules. These cathelicidin molecules are preferred to be combined with the antigen/vaccine composition according to the present invention. However, these cathelin molecules surprisingly have turned out to be also effective as an adjuvant for a antigen without the addition of further adjuvants. It is therefore possible to use such cathelicidin molecules as efficient adjuvants in vaccine formulations with or without further immunactivating substances.

The vaccine according to the present invention preferably contains as Peptide A $KLKL_5KLK$ and as I-/U-ODN oligo $d(IC)_{13}$. These two substances have shown specifically advantageous results in the experiments according to the present invention.

The vaccine according to the present invention may further (or, specifically in the case of Influenza, HCV or HBV, even instead of the U-/I-ODN) contain an oligodeoxynucleotide containing a CpG-motif as immunomodulating nucleic acids. The immunomodulating nucleic acids to be used according to the present invention can be of synthetic, prokaryotic and eukaryotic origin. In the case of eukaryotic origin, DNA should be derived from, based on the phylogenetic tree, less developed species (e.g. insects, but also others). In a preferred embodiment of the invention the immunogenic oligodeoxynucleotide (ODN) is a synthetically produced DNA-molecule or mixtures of such molecules. Derivates or modifications of ODNs such as thiophosphate substituted analogues (thiophosphate residues substitute for phosphate) as for example described in US patents US

5,723,335 and US 5,663,153, and other derivatives and modifications, which preferably stabilize the immunostimulatory composition(s) but do not change their immunological properties, are also included. A preferred sequence motif is a six base DNA motif containing an (unmethylated) CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines (5'-Pur-Pur-C-G-Pyr-Pyr-3'). The CpG motifs contained in the ODNs according to the present invention are more common in microbial than higher vertebrate DNA and display differences in the pattern of methylation. Surprisingly, sequences stimulating mouse APCs are not very efficient for human cells. Preferred palindromic or non-palindromic ODNs to be used according to the present invention are disclosed e.g. in Austrian Patent applications A 1973/2000, A 805/2001, EP 0 468 520 A2, WO 96/02555, WO 98/16247, WO 98/18810, WO 98/37919, WO 98/40100, WO 98/52581, WO 98/52962, WO 99/51259 and WO 99/56755 all incorporated herein by reference. ODNs/DNAs may be produced chemically or recombinantly or may be derived from natural sources. Preferred natural sources are insects.

The vaccine according to the present invention may preferably contain a polycationic peptide and an oligodeoxynucleotide containing a CpG-motif in combination. In the course of the present invention it has even turned out that the combination of CpG-ODN and polycationic peptide shows improvement effects in Influenza vaccine compositions, which are comparable to the effects of the combination of Peptide A and I-/U-ODNs and cannot only be combined with Peptide A and I-/U-ODNs but even be used instead of them. Of course, also mixtures of different immunostimulatory nucleic acids (I-/U-ODNs, CpG-ODNs,...) and Peptide A variants (as well as other Immunizers) may be used according to the present invention.

According to another aspect, the present invention also relates to the use of a combination of Peptide A and a I-/U-ODN, both as defined according to the present invention, to improve the protective efficacy of a

vaccine against a viral pathogen, especially influenza virus, HCV or HBV, HIV, HPV or JEV. Specifically, the antigen-specific type 1 response, especially IgG2-antibody response or IFN-gamma response, of a vaccine against a viral pathogen, especially influenza virus, HCV or HBV, HIV, HPV or JEV, can be improved and at the same time the type 2 response, especially IgG1-antibody response or interleukin 4 (IL 4) response, of said vaccine can be preserved.

It has been shown previously (WO 02/13857) that naturally occuring, cathelicidin-derived antimicrobial peptides or derivatives thereof have an immune response stimulating activity and therefore constitute highly effective type 1 inducing adjuvants (Immunizers). Main sources of antimicrobial peptides are granules of neutrophils and epithelial cells lining the respiratory, gastro-intestinal and genitourinary tracts. In general they are found at those anatomical sites most exposed to microbial invasion, are secreted into internal body fluids or stored in cytoplasmic granules of professional phagocytes (neutrophils).

In the WO 02/32451 a type 1 inducing adjuvant (Immunizer) that is able to strongly enhance the immune response to a specific co-administered antigen and therefore constitutes a highly effective adjuvant is disclosed, Peptide A comprising a sequence R_1-XZXZ$_N$XZX-R_2. A specifically preferred peptide is KLKLLLLLKLK. Besides naturally occuring antimicrobial peptides, synthetic antimicrobial peptides have been produced and investigated. The synthetic antimicrobial peptide KLKLLLLLKLK-NH_2 was shown to have significant chemotherapeutic activity in *Staphylococcus aureus*-infected mice; human neutrophils were activated to produce the superoxide anion (O_2^-) via cell surface calreticulin. The exact number and position of K and L was found to be critical for the antimicrobial activity of the synthetic peptide (Nakajima, Y. (1997); Cho, J-H. (1999)).

The present invention is especially beneficial if the combined medicament is administered, e.g. subcutaneously, intramuscularly, intradermally or transdermally. However, other application forms, such as parenteral, intravenously, intranasally, oral or topical application, are also suitable for the present invention.

The Influenza antigen may be mixed with the adjuvant (Immunizer) (composition) according to the present invention or otherwise specifically formulated e.g. as liposome, retard formulation, etc..

The vaccines according to the present invention may be administered to an individual in effective amounts known to the skilled man in the art of Influenza vaccination. Optimisation of antigen amount and Immunizer amount can be started from established amounts and using available methods.

The invention will be described in more detail by the following examples and figures, but the invention is of course not limited thereto.

Fig. 1 shows that cationic peptides co-injected with different ODNs synergistically induce strong type 1 humoral responses (IgG2b) against a commercially available Influenza-vaccine;

Fig. 2 shows that KLK/o-d$(IC)_{13}$ strongly improves the efficacy of a commercially available Influenza vaccine;

Fig. 3 shows HBsAg-specific IFN-gamma and IL-4 responses;

Fig. 4 shows the induction of a HBsAg-specific cellular type 1 response after injection of HBsAg alone or in combination with Alum and other adjuvants (Immunizers) (HBsAg-specific IFN-gamma production).

Fig. 5 shows the induction of a HBsAg-specific cellular type 2 response after injection of HBsAg alone or in

combination with Alum and other adjuvants (Immunizers) (HBsAg-specific IL-4 production).

Fig. 6 shows the induction of a HBsAg-specific humoral type 1 response after injection of HBsAg alone or in combination with Alum and other adjuvants (Immunizers) (HBsAg-specific IgG_{2b} titer).

Fig. 7 shows the induction of a HBsAg-specific humoral type 2 response after injection of HBsAg alone or in combination with Alum and other adjuvants (Immunizers) (HBsAg-specific IgG_1 titer).

Examples:

Example 1:
Cationic peptides (pR or KLK) co-injected with different oligodeoxynucleotides (ODN) (CpI, ntCpI, o-d(IC)$_{13}$) synergistically induce strong type 1 humoral responses (IgG2b) against a commercially available Influenza-vaccine (Fluvirin)

Mice	C57BL/6 (Harlan/Olac)
Influenza vaccine	Fluvirin (Evans vaccine); inactivated Influenza virus surface antigens (haemagglutinin and neuraminidase) purified of strains: A/NewCaledonia/20/99 (H1N1)-like strain (15µg haemagglutinin) A/Moscow/10/99 (H3N2)-like strain (A/Panama/2007/99 RESVIR-17) (15µg haemagglutinin) B/Sichuan/379/99-like strain (15µg haemagglutinin) dose: 1µg total protein/mouse
$Al(OH)_3$	Alhydrogel; Biosys, Denmark dose: 1:1 mixture with antigen

pR	Poly-L-Arginine with an average degree of polymerization of 43 arginine residues (determined by MALLS); Sigma Aldrich Inc dose: 100µg/mouse
KLK	KLKLLLLLKLK-COOH was synthesized by MPS (Multiple Peptide System, USA) dose: 168µg/mouse
oligo-d(IC)$_{13}$	ODN 5'ICI CIC ICI CIC ICI CIC ICI CIC IC3' was synthesized by Purimex Nucleic Acids Technology, Göttingen dose : 5nmol/mouse
I-ODN 2	thiophosphate substituted ODNs containing deoxyinosine: tcc atg aci ttc ctg atg ct, were synthesized by Purimex Nucleic Acids Technology, Göttingen dose: 5nmol/mouse
I-ODN 2b	ODNs containing deoxyinosine: tcc atg aci ttc ctg atg ct, were synthesized by Purimex Nucleic Acids Technology, Göttingen dose: 5nmol/mouse
formulation	5mM Tris/270mM Sorbitol, pH 7

experimental group (12 mice/group):

1. : naïve
2. : Flu vaccine (1µg total protein)
3. : Flu vaccine (1µg total protein) + pR
4. : Flu vaccine (1µg total protein) + KLK
5. : Flu vaccine (1µg total protein) + Al(OH)$_3$
6. : Flu vaccine (1µg total protein) + o-d(IC)$_{13}$
7. : Flu vaccine (1µg total protein) + I-ODN 2
8. : Flu vaccine (1µg total protein) + I-ODN 2b
9. : Flu vaccine (1µg total protein) + pR + I-ODN 2

10.: Flu vaccine (1µg total protein) + KLK + o-d(IC)$_{13}$
11.: Flu vaccine (1µg total protein) + KLK + I-ODN 2
12.: Flu vaccine (1µg total protein) + KLK + I-ODN 2b

On days 0, 28 and 56 C57BL/6 mice were injected s.c. into both hind footpads with a total volume of 100µl/mouse (50µl/footpad) containing the above listed compounds. Serum was collected at days 26, 54 and 82 and analyzed for Influenza vaccine-specific IgG1 and IgG2b antibodies by ELISA. Titers correspond to that dilution of serum resulting in half maximal OD_{405nm}.

Figure 1 indicates that the combined injection of cationic peptides (pR or KLK) and different ODNs (I-ODN 2, I-ODN 2b, or o-d(IC)$_{13}$) induces very potent antigen (Influenza vaccine)-specific humoral type 1 responses (IgG2b) in a synergistic way. Upon injection of Influenza vaccine alone or in combination with Al(OH)$_3$, cationic peptides (pR, KLK) only or different ODNs (except I-ODN 2) only, no specific IgG2b response is detectable. Booster vaccinations strongly increase the observed response.

Co-injection of Influenza vaccine with Al(OH)$_3$, KLK or combinations pR/I-ODN 2, KLK/I-ODN 2, KLK/I-ODN 2b or KLK/o-d(IC)$_{13}$ induces the production of Influenza vaccine-specific IgG1 (type 2 response).

Example 2:
The combination KLK/o-d(IC)$_{13}$ strongly improves the efficacy of a commercially available Influenza-vaccine (Fluvirin)

Mice	BALB/c (Harlan/Olac)
Influenza vaccine	Fluvirin (Evans vaccine); inactivated Influenza virus surface antigens (haemagglutinin and neuraminidase) purified of strains: A/NewCaledonia/20/99 (H1N1)-like strain (15µg haemagglutinin)

	A/Moscow/10/99 (H3N2)-like strain (A/Panama/2007/99 RESVIR-17) (15µg haemagglutinin) B/Sichuan/379/99-like strain (15µg haemagglutinin) dose: 1µg total protein/mouse (= low dose / literature: 10µg/mouse)
$Al(OH)_3$	Alhydrogel; Biosys, Denmark dose: 1:1 mixture with antigen
KLK	KLKLLLLLKLK-COOH was synthesized by MPS (Multiple Peptide System, USA) dose: 168µg/mouse
oligo-$d(IC)_{13}$	ODN 5'ICI CIC ICI CIC ICI CIC ICI CIC IC3' was synthesized by Purimex Nucleic Acids Technology, Göttingen dose : 5nmol/mouse
formulation	5mM Tris/270mM Sorbitol, pH 7

<u>experimental group</u> (12 mice/group):

1. naïve
2. Flu vaccine (1µg total protein)
3. Flu vaccine (1µg total protein) + $Al(OH)_3$
4. Flu vaccine (1µg total protein) + KLK + o-$d(IC)_{13}$

On days 0, 28 and 56 BALB/c mice were injected s.c. into both hind footpads with a total volume of 100µl/mouse (50µl/footpad) containing the above listed compounds. Serum was collected at days 26, 54 and 82 and analyzed for neutralizing anti-haemagglutinin antibodies by using a standard haemagglutination inhibition assay. Briefly, the presence of haemagglutinin on the virus surface induces haemagglutination of erythrocytes, which can be inhibited by neutralizing anti-haemagglutinin antibodies. Titers of antibodies against haemagglutinin of the different viral strains (A1 = A/NewCaledonia/20/99 (H1N1)-like strain; A2 = A/Panama/2007/99 RESVIR-17; B =

B/Sichuan/379/99-like strain) were determined. Titer of serum corresponds to end point dilution showing inhibition.

In contrast to injection of Influenza vaccine alone or in combination with $Al(OH)_3$ the co-injection of Influenza vaccine plus KLK and $o\text{-}d(IC)_{13}$ induces high levels of neutralizing antibodies against all three tested haemagglutinins (Fig. 2). Since effectiveness of an Influenza vaccine has been shown to correlate with serum titers of anti-haemagglutinin antibodies the obtained results indicate a high potential of KLK/$o\text{-}d(IC)_{13}$ for the induction of protection against Influenza.

Example 3:
Cationic peptides (pR or KLK) co-injected with different oligodeoxynucleotides (ODN) (CpI, ntCpI, $o\text{-}d(IC)_{13}$) synergistically induce strong type 1 cellular responses (IFN-gamma) against Hepatitis B surface Antigen

Mice	C57Bl/6 (Harlan-Winkelmann, Germany); low responder mice for HbsAg-specific immune responses
Antigen	Hepatitis B surface antigen (HBsAg) dose: 5µg / mouse
$Al(OH)_3$	Alhydrogel; Biosys, Denmark dose: 1:1 mixture with antigen
pR	Poly-L-Arginine with an average degree of polymerization of 43 arginine residues (determined by MALLS); Sigma Aldrich Inc dose: 100µg /mouse

KLK	KLKLLLLLKLK-COOH was synthesized by MPS (Multiple Peptide System, USA) dose: 168µg / mouse
I-ODN 2	(= CpI 2) thiophosphate substituted ODNs containing deoxyinosines: 5'tcc atg aci ttc ctg atg ct 3' were synthesized by Purimex Nucleic Acids Technology, Göttingen dose: 5nmol / mouse
I-ODN 2b	(= CpI 2b) ODNs containing deoxyinosines: 5'tcc atg aci ttc ctg atg ct 3' were synthesized by Purimex Nucleic Acids Technology, Göttingen dose: 5nmol / mouse
o-d(IC)$_{13}$	ODN 5'ICI CIC ICI CIC ICI CIC ICI CIC IC3' was synthesized by Purimex Nucleic Acids Technology, Göttingen dose: 5nmol / mouse
formulation	5mM Tris/270mM Sorbitol, pH 7

<u>experimental group</u> (5 mice/group/timepoint):

1. : HBsAg
2. : HBsAg + Alum
3. : HBsAg + I-ODN 2
4. : HBsAg + I-ODN 2b
5. : HBsAg + o-d(IC)$_{13}$
6. : HBsAg + pR

7. : HBsAg	+ KLK	
8. : HBsAg	+ pR	+ I-ODN 2
9. : HBsAg	+ pR	+ I-ODN 2b
10.: HBsAg	+ pR	+ o-d(IC)$_{13}$
11.: HBsAg	+ KLK	+ I-ODN 2
12.: HBsAg	+ KLK	+ I-ODN 2b
13.: HBsAg	+ KLK	+ o-d(IC)$_{13}$

On day 0 and day 56 mice were injected subcutaneously into the right flank with a total volume of 100µl/mouse containing the above mentioned compounds. The analysis of the immune response was performed at day 7, day 21 and day 50 after first and second injection, respectively. Spleen cells of five mice per group per time point were restimulated *ex vivo* with 10µg/ml HBsAg and ELISPOT assays were performed in order to analyse the HBsAg-specific IFN-gamma (type 1 immune response) as well as IL-4 (type 2 immune response) production.

Fig. 3 HBsAg injected alone or in combination with Alum induces no or only very low levels of IFN-gamma, whereas upon injection of HBsAg combined with pR/ODN or KLK/ODN an HBsAg-specific IFN-gamma production is induced which can be further increased by booster vaccination. Slightly increased IL-4 production compared to injection of HBsAg alone is observable upon co-injection of Alum, pR and KLK after boost, as well upon co-injection of KLK/ODN combinations.

Example 4:

Herein, an example is provided, which shows that upon co-injection of the Hepatitis B surface Antigen (HBsAg), various type 1 inducing adjuvant compositions according ot the present invention and Alum the type 1 response induced by the type 1 inducing adjuvants (Immunizers) is strongly increased at least after boost when compared to injection of HBsAg/Immunizer alone. However, the Alum-induced type 2 response is not affected.

Materials and Methods:

Mice	C57Bl/6 (Harlan-Winkelmann, Germany); low responder mice for HbsAg-specific immune responses 5 mice/group/timepoint
Antigen	Hepatitis B surface antigen (HBsAg) dose: 5µg/mouse
poly-L-arginine	poly-L-arginine with an average degree of polymerisation of 43 arginine residues; Sigma chemicals dose: 100µg/mouse
KLK	KLKLLLLLKLK-COOH was synthesized by MPS (Multiple Peptide System, USA) Dose: 168µg/mouse
I-ODN 2	thiophosphate substituted ODNs containing deoxyinosines: 5'tcc atg aci ttc ctg atg ct3' were synthesized by Purimex Nucleic Acids Technology, Göttingen Dose: 5nmol/mouse
I-ODN 2b	ODNs containing deoxyinosines: 5'tcc atg aci ttc ctg atg ct 3' were synthesized by Purimex Nucleic Acids Technology, Göttingen Dose: 5nmol/mouse
o-d(IC)$_{13}$	ODN 5'ICI CIC ICI CIC ICI CIC ICI CIC IC3' was synthesized by Purimex Nucleic Acids Technology, Göttingen Dose: 5nmol/mouse

Exp A:

1. HBsAg
2. HBsAg + Alum
3. HBsAg + I-ODN 2
4. HBsAg + I-ODN 2b

5. HBsAg + o-d(IC)$_{13}$
6. HBsAg + pR
7. HBsAg + KLK
8. HBsAg + pR + I-ODN 2
9. HBsAg + pR + I-ODN 2b
10. HBsAg + pR + o-d(IC)$_{13}$
11. HBsAg + KLK + I-ODN 2
12. HBsAg + KLK + I-ODN 2b
13. HBsAg + KLK + o-d(IC)$_{13}$

Exp B:

1. HbsAg/Alum
2. HbsAg/Alum + I-ODN 2
3. HbsAg/Alum + I-ODN 2b
4. HbsAg/Alum + o-d(IC)$_{13}$
5. HbsAg/Alum + pR
6. HBsAg/Alum + KLK
7. HbsAg/Alum + pR + I-ODN 2
8. HbsAg/Alum + pR + I-ODN 2b
9. HbsAg/Alum + pR + o-d(IC)$_{13}$
10. HBsAg/Alum + KLK + I-ODN 2
11. HBsAg/Alum + KLK + I-ODN 2b
12. HbsAg/Alum + KLK + o-d(IC)$_{13}$

On day 0 and day 56 mice were injected subcutaneously into the right flank with a total volume of 100µl/mouse containing the above mentioned compounds. The analysis of the immune response was performed at (day 7) day 21 and day 50 after first and second injection, respectively. Spleen cells of five mice per group per time point were restimulated ex vivo with 10µg/ml HBsAg and ELISPOT assays were performed in order to analyse the HBsAg-specific IFN-gamma (type 1 immune response) as well as IL-4 (type 2 immune response) production. Furthermore, serum was taken at the indicated time points and the HBsAg-specific IgG_{2b} (type 1 immune response) as well as IgG_1 (type 2 immune response) titers were determined.

Results:

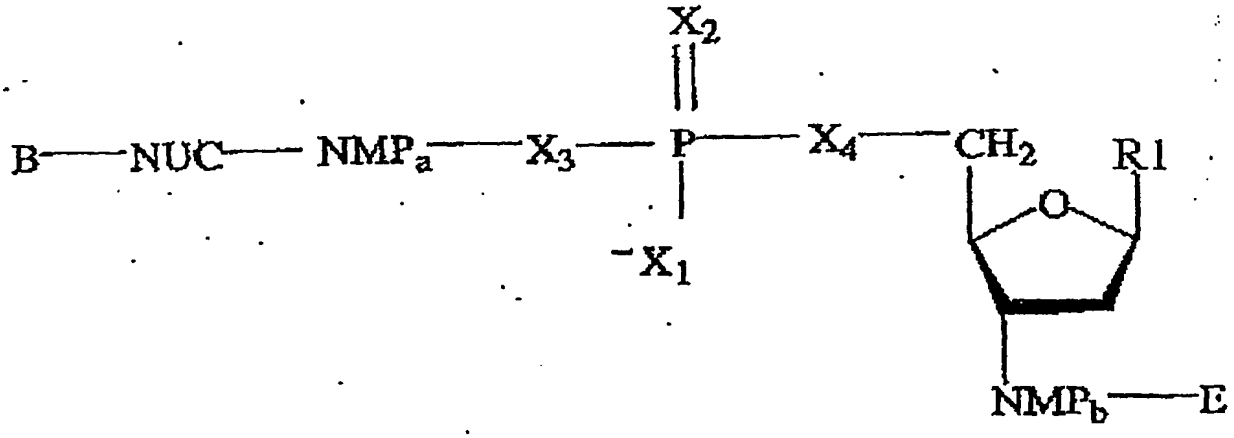
Fig. 4: Induction of a HBsAg-specific cellular type 1 response (HBsAg-specific IFN-gamma production)
HBsAg injected alone or in combination with Alum induces no or only very low levels of IFN-gamma, whereas upon injection of HBsAg combined with the different Immunizers (pR/ODN, KLK/ODN) an HBsAg-specific IFN-gamma production is induced which can be further increased by booster vaccination (Exp. A). However, upon co-injection of HBsAg/Immunizer and Alum the induced IFN-gamma production after boost is strongly increased (Exp. B).
Fig. 5: Induction of a HBsAg-specific cellular type 2 response (HBsAg-specific IL-4 production)
HBsAg injected in combination with Alum induces HBsAg-specific IL-4 production, which is not further affected by the co-injection of the different Immunizers (Exp. B).
Fig. 6: Induction of a humoral type 1 response (HBsAg-specific IgG2b titer)
HBsAg injected alone or in combination with Alum induces no HBsAg-specific IgG2b, whereas upon injection of HBsAg combined with the different pR/ODN-based Immunizers potent IgG2b titers are detectable after boost (Exp. A). The co-injection of Alum has no real influence on these titers (Exp. B). Upon injection of HBsAg/KLK-ODN-based Immunizer no antibody titers are induced at all (Exp. A, B).
Fig. 7: Induction of a humoral type 2 response (HBsAg-specific IgG1 titer)
HBsAg injected in combination with Alum induces HBsAg-specific IgG1 titer, which are not further affected by the co-injection of the pR/ODN-based Immunizer (Exp. B). Upon use of KLK-ODN-based Immunizer no antibody titers are induced at all (Exp. A, B).

Conclusions:
Compared to the injection of antigen with the combination of Immunizers according to the present invention, the co-injection of Immunizers with Alum induce enhanced cellular type 1 immune responses (IFN-gamma), while the Alum-induced type 2 response (IL-4) is not affected. This observation makes the Immunizers very attractive in at least two ways. On the one hand, existing Alum-based

vaccines can be improved by type 1 inducing Immunizers, e.g. in order to induce cell mediated type 1 responses which were lacking so far for special applications like therapeutic vaccines against viral infections. On the other hand, more potent type 1 responses can be induced in general when the combination Immunizer/Alum is used as vaccine adjuvant.

Claims:

1. Vaccine for preventing infections of influenza virus comprising
- an antigen,
- a peptide comprising a sequence R_1-XZXZ$_N$XZX-R_2, whereby N is a whole number between 3 and 7, preferably 5, X is a positively charged natural and/or non-natural amino acid residue, Z is an amino acid residue selected from the group consisting of L, V, I, F and/or W, and R_1 and R_2 are selected independantly one from the other from the group consisting of -H, -NH_2, -$COCH_3$, -COH, a peptide with up to 20 amino acid residues or a peptide reactive group or a peptide linker with or without a peptide; X-R_2 may be an amide, ester or thioester of the C-terminal amino acid residue of the peptide ("Peptide A"), and
- an immunostimulatory oligodeoxynucleic acid molecule (ODN) having the structure according to the formula (I)



wherein
R1 is selected from hypoxanthine and uracile,
any X is O or S,
any NMP is a 2' deoxynucleoside monophosphate or monothiophosphate, selected from the group consisting of deoxyadenosine-, deoxyguanosine-, deoxyinosine-, deoxycytosine-, deoxyuridine-, deoxythymidine-, 2-methyl-deoxyinosine-, 5-methyl-deoxycytosine-, deoxypseudouridine-, deoxyribosepurine-, 2-amino-deoxyribosepurine-, 6-S-deoxyguanine-, 2-dimethyl-deoxyguanosine- or N-isopentenyl-deoxyadenosine-monophosphate or -monothiophosphate,
NUC is a 2' deoxynucleoside, selected from the group consisting of deoxyadenosine-, deoxyguanosine-, deoxyinosine-, deoxycytosine-, deoxyinosine-, deoxythymidine-, 2-methyl-deoxyuridine-, 5-methyl-deoxycytosine-, deoxypseudouridine-, deoxyribosepurine-, 2-amino-deoxyribosepurine-, 6-S-deoxyguanine-, 2-dimethyl-deoxyguanosine- or N-isopentenyl-deoxyadenosine,
a and b are integers from 0 to 100 with the proviso that a + b is between 4 and 150, and
B and E are common groups for 5' or 3' ends of nucleic acid molecules ("I-/U-ODN").

2. Vaccine according to claim 1, characterised in that it further contains an $Al(OH)_3$ adjuvant.

3. Vaccine according to claim 1 or 2, characterised in that said antigen is a viral antigen, preferably an influenza, especially a haemagglutinin antigen or a neuraminidase antigen, HCV or HBV, HIV, HPV or JEV antigen, a combined antigen or a combination of one or more of these antigens.

4. Vaccine according to any one of claims 1 to 3, characterised in that it further contains a polycationic peptide.

5. Vaccine according to any one of claims 1 to 4, characterised in that said Peptide A is $KLKL_5KLK$ and said I-/U-ODN is oligo $d(IC)_{13}$.

6. Vaccine according to any one of claims 1 to 5, characterised in that it further contains an oligodeoxynucleotide containing a CpG-motif.

7. Vaccine according to any one of claims 1 to 6, characterised in that it further contains a polycationic peptide and an oligodeoxynucleotide containing a CpG-motif.

8. Use of a combination of Peptide A and a I-/U-ODN, both as defined in claim 1, to improve the protective efficacy of a vaccine against viral infection, especially against an infection with influenza virus, HBV, HCV, HPV, HIV or JEV.

9. Use of a combination of Peptide A and a I-/U-ODN, both as defined in claim 1, to improve the antigen-specific type 1 response, especially IgG2-antibody response or IFN-gamma response, of a vaccine against viral infections, especially infections with influenza virus, HBV, HCV, HIV, HPV or JEV, and at the same time preserving the type 2 response, especially IgG1-antibody response or interleukin 4 (IL 4) response, of said vaccine.

Summary:

Improved Vaccines

The invention refers to an improved vaccine against infections with pathogens, especially viral pathogens, comprising an antigen, a peptide of the formula R_1-$XZXZ_NXZX$-R_2 and an immunostimulatory deoxynucleic acids containing deoxyinosine and/or deoxyuridine residues.

Fig. 1 cationic peptides (pR or KLK) co-injected with different ODN (CpI, ntCpI, o-d(IC)$_{13}$) synergistically induce strong type 1 humoral responses (IgG2b) against a commercially available Influenza-vaccine (Fluvirin)

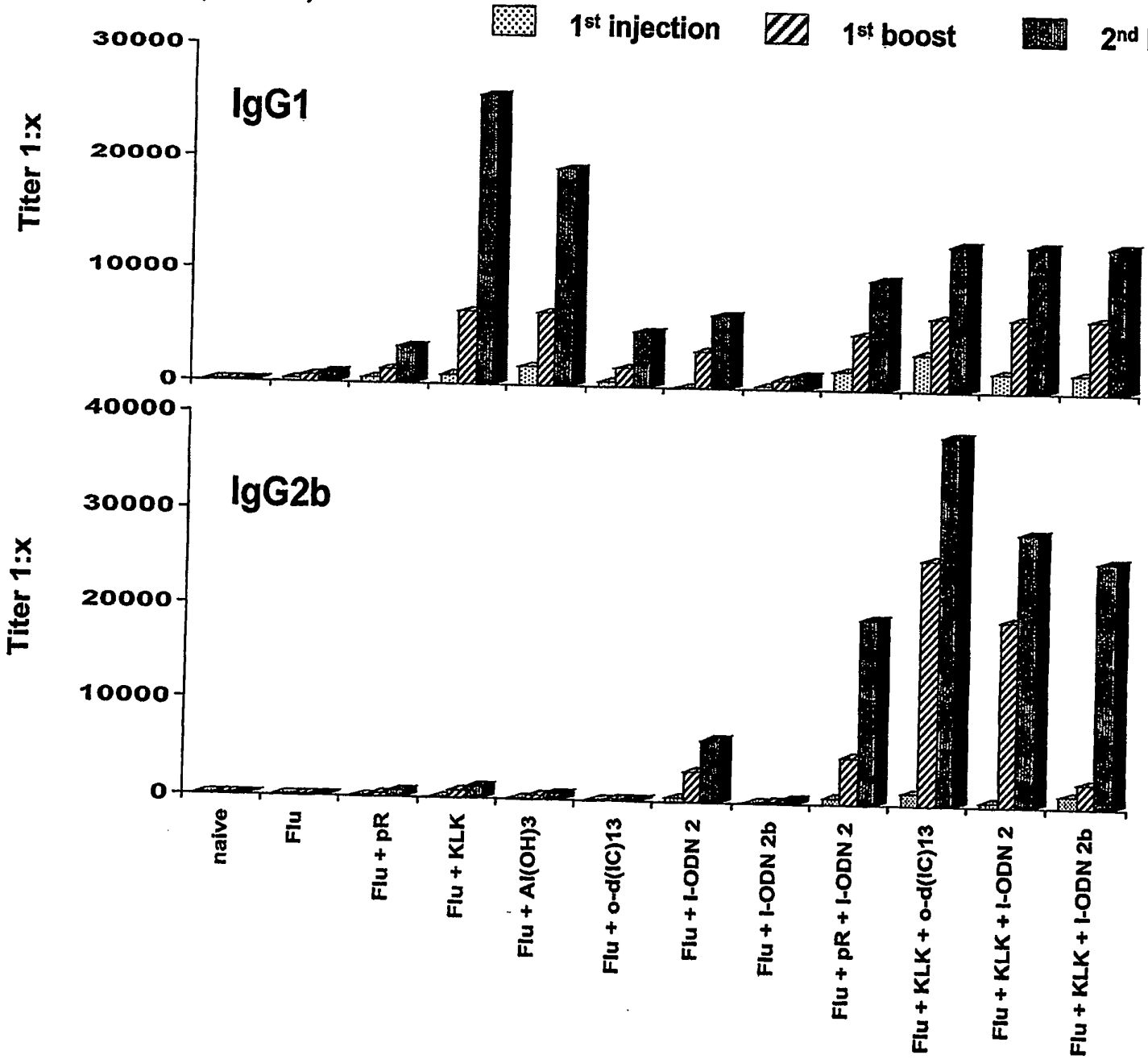


Fig. 2 KLK/o-d(IC)$_{13}$ strongly improves the efficacy of a commercially available Influenza vaccine (Fluvirin)

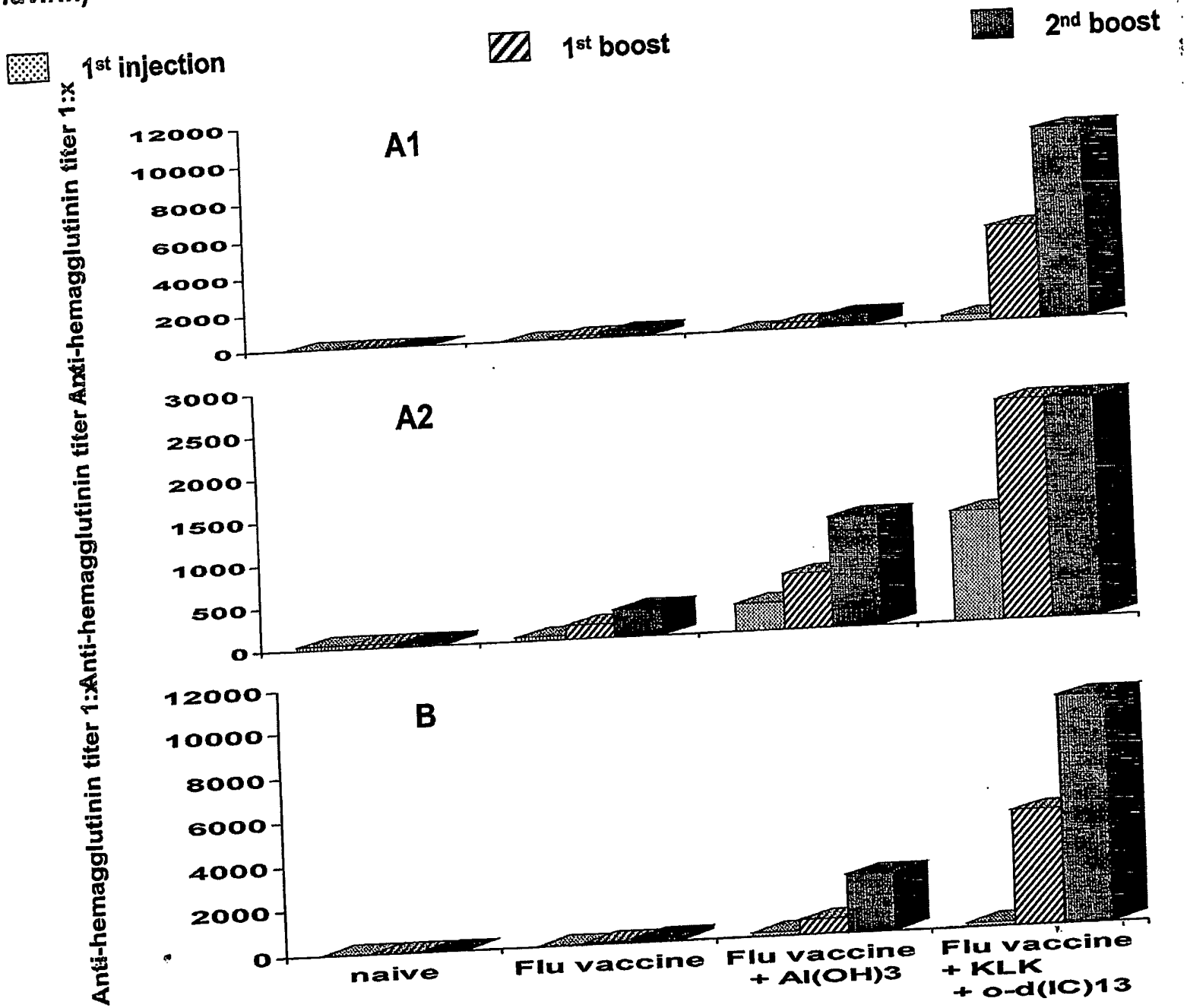
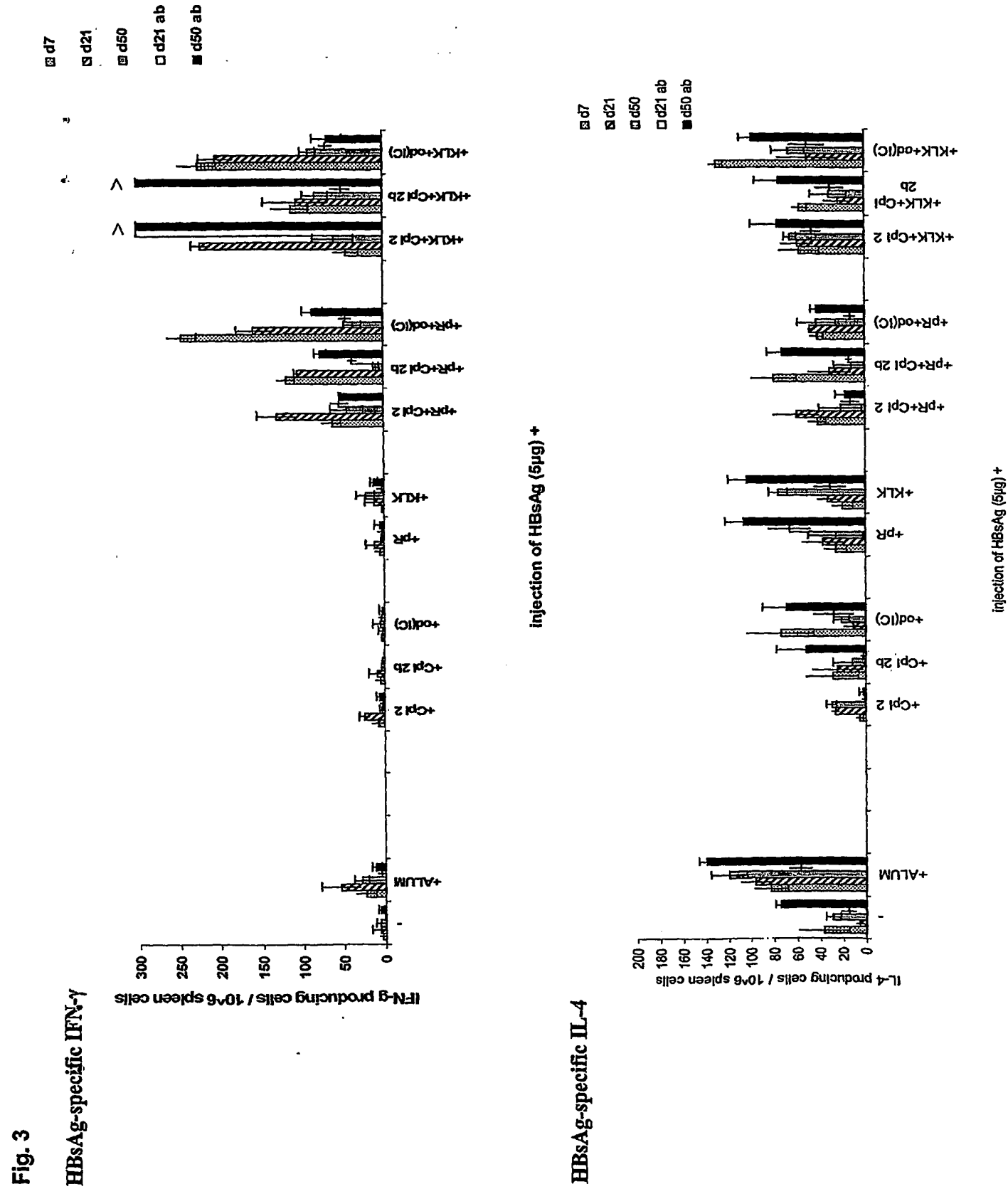


Fig. 3


HBsAg-specific IFN-γ
IFN-g producing cells / 10^6 spleen cells
d7
d21
d50
d21 ab
d50 ab
-
+ALUM
+Cpl 2
+Cpl 2b
+od(IC)
+pR
+KLK
+pR+Cpl 2
+pR+Cpl 2b
+pR+od(IC)
+KLK+Cpl 2
+KLK+Cpl 2b
+KLK+od(IC)
injection of HBsAg (5µg) +
HBsAg-specific IL-4
IL-4 producing cells / 10^6 spleen cells
injection of HBsAg (5µg) +

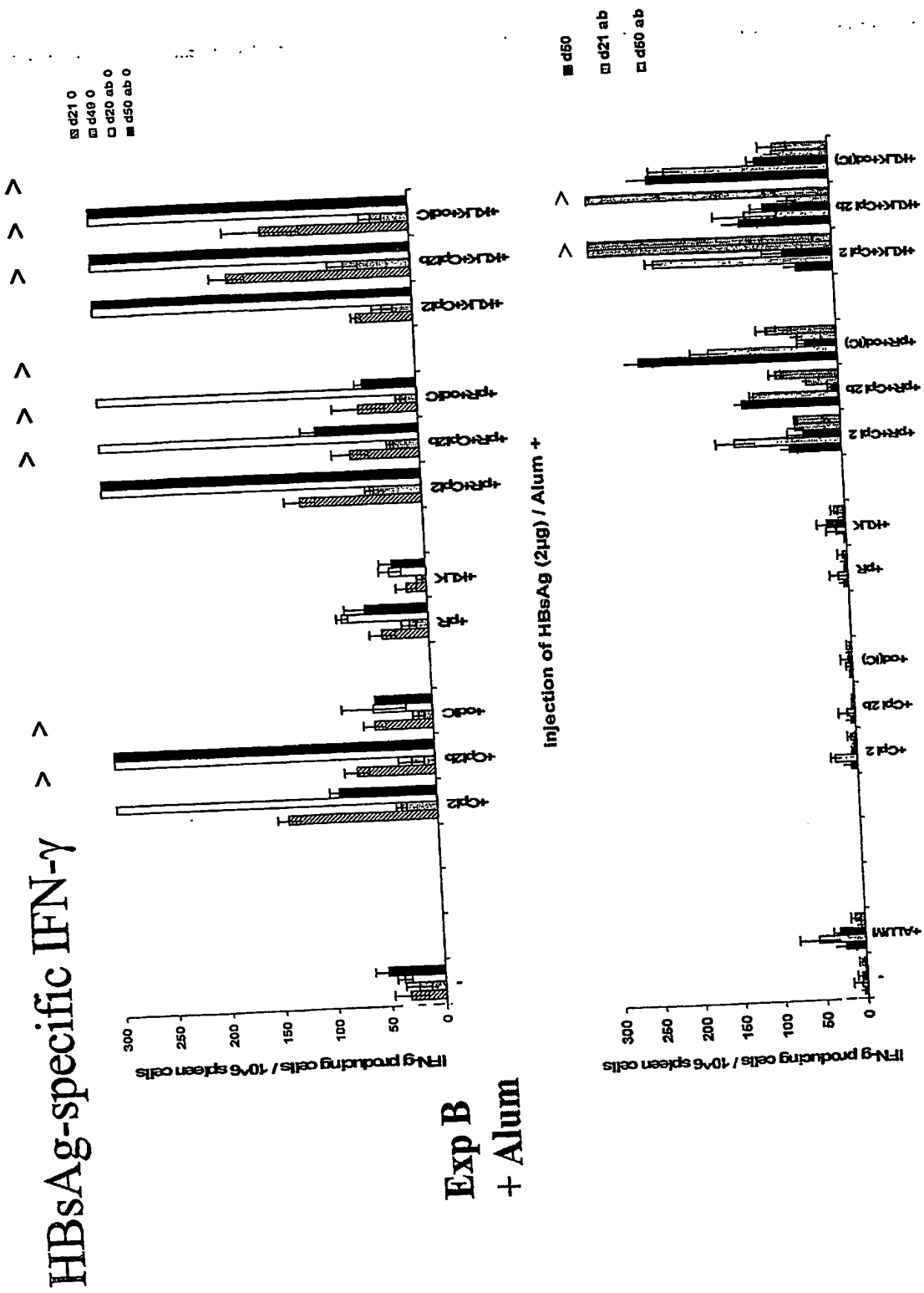

HBsAg-specific IFN-γ
Exp B
+ Alum
d21 0
d49 0
d20 ab 0
d50 ab 0
IFN-g producing cells / 10^6 spleen cells
injection of HBsAg (2µg) / Alum +
+CpG2
+CpG2b
+odIC
+PR
+ALK
+PR+CpG2
+PR+CpG2b
+PR+odIC
+ALK+CpG2
+ALK+CpG2b
+ALK+odIC
Exp A
d50
d21 ab
d50 ab
+ALUM
+CpG 2
+CpG 2b
+odI(C)
+PR+CpG 2
+PR+CpG 2b
+PR+odI(C)
+ALK+CpG 2
+ALK+CpG 2b
+ALK+odI(C)
injection of HBsAg (5µg) +

Fig. 4

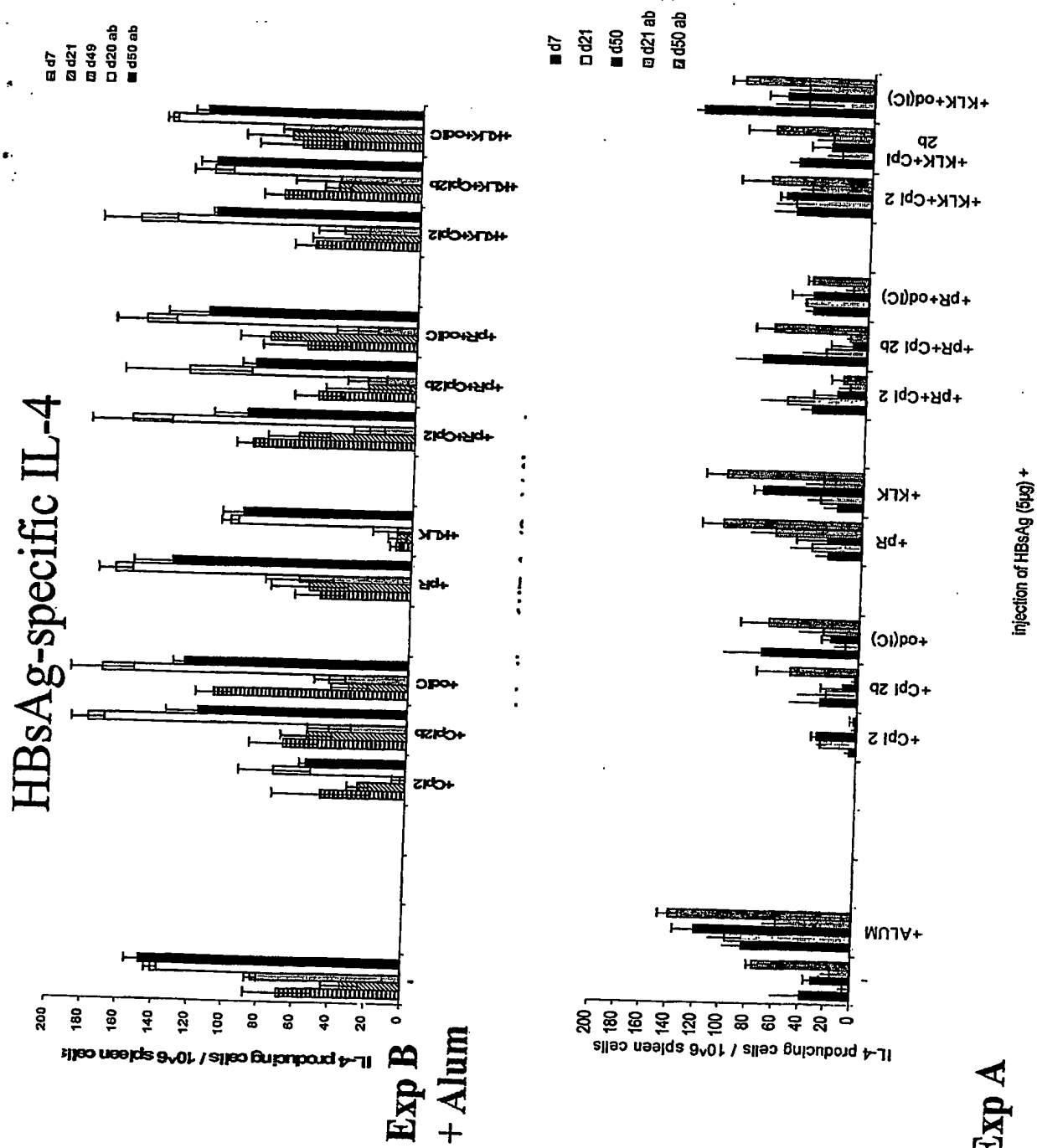

HBsAg-specific IL-4
Exp B
+ Alum
IL-4 producing cells / 10^6 spleen cells
d7
d21
d49
d20 ab
d50 ab
+cpi2
+cpi2b
+odic
+pR
+kLK
+pR+cpi2
+pR+cpi2b
+pR+odic
+kLK+cpi2
+kLK+cpi2b
+kLK+odic
Exp A
IL-4 producing cells / 10^6 spleen cells
d7
d21
d50
d21 ab
d50 ab
+ALUM
+Cpi 2
+Cpi 2b
+od(IC)
+pR
+KLK
+pR+Cpi 2
+pR+Cpi 2b
+pR+od(IC)
+KLK+Cpi 2
+KLK+Cpi 2b
+KLK+od(IC)
injection of HBsAg (5µg) +

Fig. 5

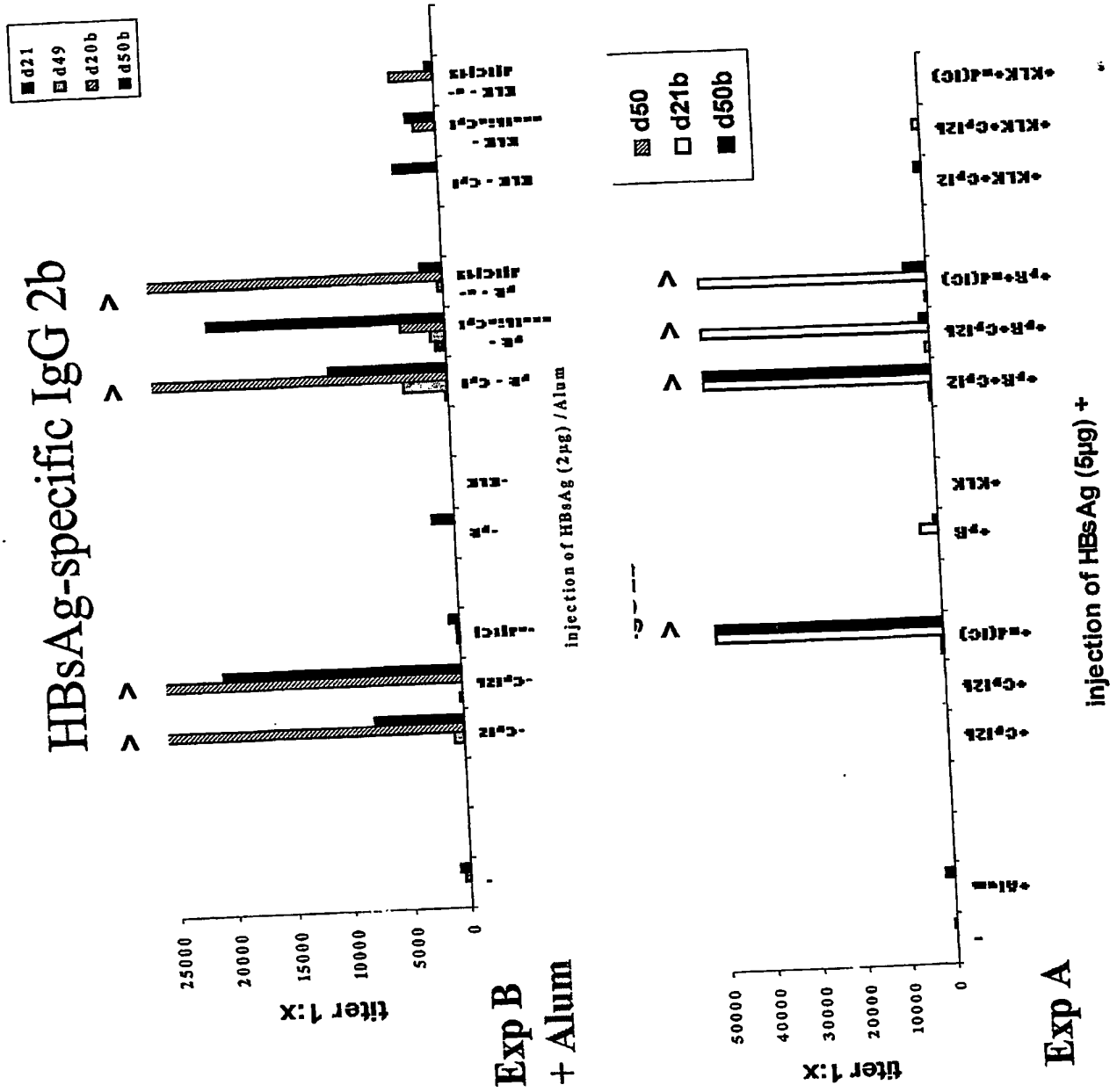

HBsAg-specific IgG 2b
d21
d49
d20b
d50b
titer 1:x
injection of HBsAg (2µg) /Alum
Exp B
+ Alum
d50
d21b
d50b
titer 1:x
injection of HBsAg (5µg) +
Exp A

Fig. 6

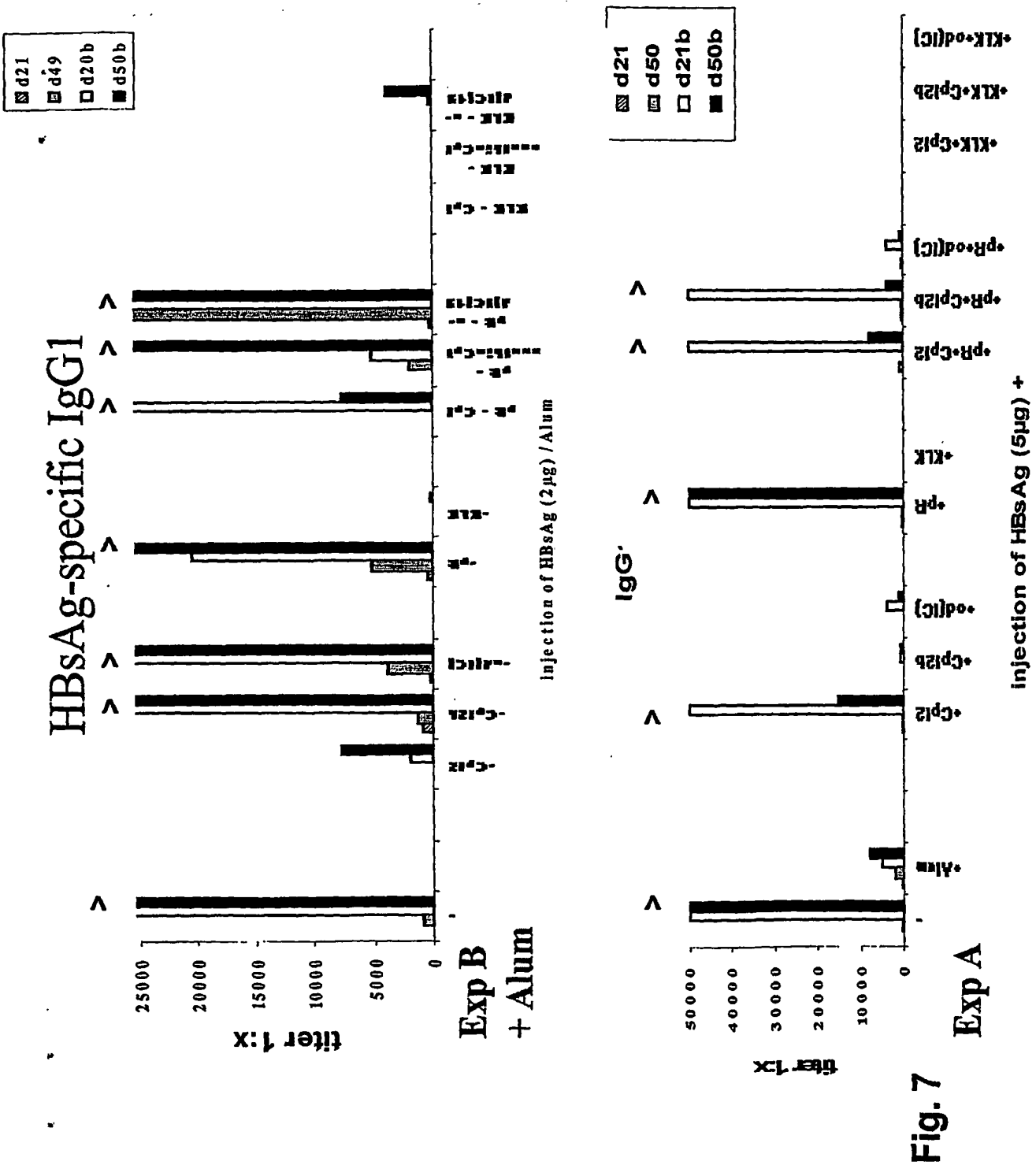

HBsAg-specific IgG1
d21
d49
d20b
d50b
titer 1:x
25000
20000
15000
10000
5000
0
Exp B
+ Alum
injection of HBsAg (2µg) / Alum
IgG
d21
d50
d21b
d50b
titer 1x
50000
40000
30000
20000
10000
0
Exp A
injection of HBsAg (5µg) +

Fig. 7

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